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Anaerobic Cellulolytic Bacteria from Wetwood of Living Trees

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Obligately anaerobic, mesophilic, cellulolytic bacteria were isolated from the wetwood of elm and maple trees. The isolation of these bacteria involved inoculation of selective enrichment cultures with increment cores taken from trees showing evidence of wetwood. Cellulolytic bacteria were present in the cores from seven of nine trees sampled, as indicated by the disappearance of cellulose from enrichment cultures. With two exceptions, cellulolytic activity was confined to the darker, wetter, inner section of the cores. Cellulolytic bacteria were also present in the fluid from core holes. The cellulolytic isolates were motile rods that stained gram negative. Endospores were formed by some strains. The physiology of one of the cellulolytic isolates (strain JW2) was studied in detail. Strain JW2 fermented cellobiose, D-glucose, glycerol, L-arabinose, D-xylose, and xylan in addition to cellulose. In a defined medium, *p*-aminobenzoic acid and biotin were the only exogenous growth factors required by strain JW2 for the fermentation of cellobiose or cellulose. Acetate and ethanol were the major nongaseous end products of cellulose fermentation. The guanine-plus-cytosine content of the DNA of strain JW2 was 33.7 mol%. Cellulolytic bacteria have not previously been reported to occur in wetwood. The isolation of such bacteria indicates that cellulolytic bacteria are inhabitants of wetwood environments and suggests that they may be involved in wetwood development.

Wetwood is an abnormal condition of the heartwood of trees (5). It occurs in a variety of trees, including both broadleaf trees and conifers, but it is most prevalent in species of elm (16). The xylem tissues of wetwood have a high water content and appear darker and wetter than does adjacent sapwood. A wide variety of obligately and facultatively anaerobic bacteria have been isolated from wetwood, including pectin-degrading bacteria, nitrogen-fixing bacteria, clostridia, and methanogens (21, 22, 24, 27, 28). Although the cause(s) of wetwood formation in trees is not known, at least some of the characteristics of the wetwood syndrome may be attributed to the activity of pectin-degrading bacteria, inasmuch as wetwood contains large numbers of pectinolytic bacteria and pectate lyase activity (23).

Wetwood is an environment in which anaerobic cellulolytic bacteria might be expected to thrive. However, cellulolytic bacteria have not been reported to occur in the wetwood of living trees. The objectives of the present study were to attempt the isolation of anaerobic cellulolytic bacteria from wetwood and to compare any cellulolytic isolates obtained from wetwood with cellulolytic bacteria from other natural environments.

(A preliminary report of part of this work has been presented [J. E. Warshaw, S. B. Leschine, and E. Canale-Parola, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, I8, p. 122].)

MATERIALS AND METHODS

Media, culture conditions, and bacterial strains. All media were prereduced (10), and all cultures were incubated anaerobically in an atmosphere of N_2 at 30°C unless specified otherwise. Cellulolytic strains were routinely cultured in RFT-C broth (12) which contained 0.6% (dry wt/vol) cellulose (ball-milled filter paper) as fermentable substrate. RFT-C soft agar, RFT-CB broth and agar media which contained cellobiose (0.5% [wt/vol]) as fermentable substrate, and RFT broth and agar which lacked fermentable substrate were prepared as previously described (12). Before use, plates of agar media were stored in an atmosphere of 5% CO_2–10% H_2–85% N_2 within an anaerobic chamber (Coy Laboratory Products, Inc., Ann Arbor, Mich.) until the resazurin indicator became colorless. Chemically defined media MJ-CB and MJ-C were used to determine growth factor requirements. Medium MJ-CB was the same as medium MJ of Johnson et al. (11), except that the vitamins were omitted. Medium MJ-C was prepared in the same way as medium MJ-CB, except that cellobiose was omitted and cellulose (ball-milled filter paper; 0.6% [wt/vol] final concentration) was added to the medium before sterilization by autoclaving.

Clostridium papyrosolvens (13) was obtained from the National Collection of Industrial Bacteria, Aberdeen, Scotland (NCIB 11394), and strain C7 (12) was from our laboratory culture collection.

Enrichment and isolation of cellulolytic bacteria. Trees showing external evidence of wetwood (16) were sampled 1 m above ground level with a sterile increment borer (4.3 by 160 mm). Samples from seven American elms (*Ulmus americana*), one sugar maple (*Acer saccharum*), and one silver maple (*Acer saccharinum*) were studied. Trees were located on the campus of the University of Massachusetts or in surrounding fields and woodlands. Wood cores and liquid from core holes were collected by the method of Schink et al. (22) and placed in sterile neoprene-stoppered anaerobic culture tubes (25 by 142 mm; Bellco Glass, Inc., Vineland, N.J.) containing N_2. Each core was removed from the collection tube with sterile forceps, placed in a sterile petri dish, and cut into three approximately equal sections with a sterile single-edged razor blade, thus separating the wetwood which is the darker, wetter, inner portion of the core, from the sapwood, which is the outermost living tissue. Enrichment cultures were prepared by transferring each wood core section or ca. 0.05 ml of fluid from the core hole into 5 ml of RFT-C broth contained in a neoprene-stoppered culture tube.

To isolate strains of cellulolytic bacteria, liquid and sediment from enrichment cultures showing cellulose disappearance were serially diluted in RFT broth. Melted RFT-C soft agar medium (5 ml at 45°C) was inoculated with 0.2 ml of

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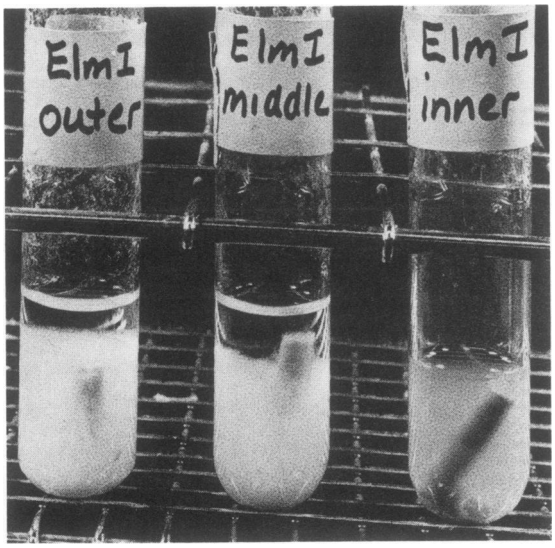

FIG. 1. Enrichment cultures inoculated with the outer, middle, and inner section of a wood core from an American elm. After 14 days of incubation, cellulose had disappeared from the culture that had been inoculated with the inner section of the core, while the other cultures remained opaque due to the presence of cellulose fibers in the medium.

diluted enrichment culture, and the inoculated soft agar was poured evenly onto RFT agar plates in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, Mich.). Due to the presence of cellulose fibers in the soft agar overlay, the medium in the plates initially appeared opaque. However, within 3 to 13 days, clear zones appeared in the soft agar around colonies of cellulose-fermenting bacteria. To obtain pure cultures, cells were removed from colonies with a sterile loop, transferred by streaking onto plates of RFT-CB agar medium in the anaerobic chamber, and restreaked several times. Cells from isolated colonies were then transferred into tubes of RFT-C broth to determine whether the isolate was cellulolytic. By means of this procedure, cellulolytic strains JW2, JW3, and JW4 were isolated.

Nutritional characteristics. The ability of strain JW2 to utilize various soluble compounds as fermentable substrates was determined by estimating visually the turbidity of cultures after three transfers in RFT broth medium containing the potential substrate, as previously described (12). The ability to ferment various sources of cellulose in RFT broth was determined by measuring the amount of ethanol in culture supernatant fluid as previously described (12). Growth factor requirements for cellulose or cellobiose fermentation were determined in defined media MJ-C and MJ-CB, respectively. These media were first supplemented with a growth factor mixture (8) that supported the fermentation of cellulose in medium MJ-C (as determined by noting the disappearance of cellulose from cultures) and cellobiose in medium MJ-CB (as determined by estimating visually the turbidity of cultures). Growth factors were then omitted from these media one at a time to determine which factors were required.

Analysis of fermentation products. Gas chromatography methods used for qualitative analysis of volatile and nonvolatile fatty acid fermentation products were described previously (6). Ethanol production was assayed enzymatically with alcohol dehydrogenase (Ethyl Alcohol Reagent Set; Worthington Diagnostics, Freehold, N.J.).

Microscopy. Cells examined by phase-contrast and electron microscopy were grown in RFT-CB broth for 24 h. Three-day-old RFT-C broth cultures were used to inoculate the RFT-CB broth (0.5 ml of inoculum added to 10 ml of RFT-CB broth). Methods and equipment used for phase-contrast photomicroscopy (26) and for transmission electron microscopy (18) were described previously.

Determination of guanine-plus-cytosine content of DNA. DNA was purified by the method of Marmur (15). The guanine-plus-cytosine content of the purified DNA was determined from its thermal denaturation temperature by the method of Mandel and Marmur (14), with *Escherichia coli* K-12 DNA as a standard.

Chemicals and cellulosic substrates. All chemicals were reagent grade. Avicel microcrystalline cellulose type PG-105 was a gift of FMC Corp., Philadelphia, Pa.; absorbent cotton (USP grade) was purchased from Absorbant Cotton Co., Valley Park, Mich.; nonabsorbent cotton coil (for 18- to 20-mm tubes) was from American Scientific Products, McGraw Park, Ill.; and Solka-Floc was from Brown Co., Berlin, N.H.

RESULTS

Enrichment and isolation of cellulolytic bacteria. Strains of cellulolytic bacteria were isolated from wood core samples or core hole fluid samples that were taken from trees showing external evidence of wetwood. Figure 1 shows a set of enrichment cultures that had been inoculated with sections of a wood core from an American elm. After 14 days of incubation, only the culture inoculated with the inner section of the core showed evidence of cellulose degradation, indicating that cellulolytic microorganisms were present in that section of the core. The inner section of the increment core from seven of nine trees sampled contained cellulolytic microorganisms; with two exceptions, cellulolytic activity was confined to the inner section of the core (Table 1). Cellulolytic bacteria were also present in the liquid from the core holes of the two maples sampled (Table 1).

Cellulolytic bacterial strains JW2 and JW3 were isolated from the wetwood of an American elm and a sugar maple, respectively. Strain JW4 was isolated from core hole fluid of a silver maple.

Cell morphology. The cells of strain JW2 (Fig. 2A) were curved motile rods with an average length of approximately

TABLE 1. Cellulolytic activity of samples of wetwood[a]

Sample	Cellulolytic activity of:			
	Increment core section			Liquid from core hole
	Outer	Middle	Inner	
American elm	−	−	+	NS
American elm	+	−	+	NS
American elm	−	−	+	NS
American elm	−	−	+	NS
American elm	−	−	−	NS
American elm	−	−	+	NS
American elm	−	−	−	NS
Silver maple	−	−	+	+
Sugar maple	−	+	+	+

[a] Cellulolytic activity was indicated by the disappearance of cellulose from enrichment cultures inoculated with a section of an increment wood core or with liquid from the core hole (see Materials and Methods). +, Presence of cellulolytic bacteria; −, absence of cellulolytic bacteria; NS, liquid not sampled.

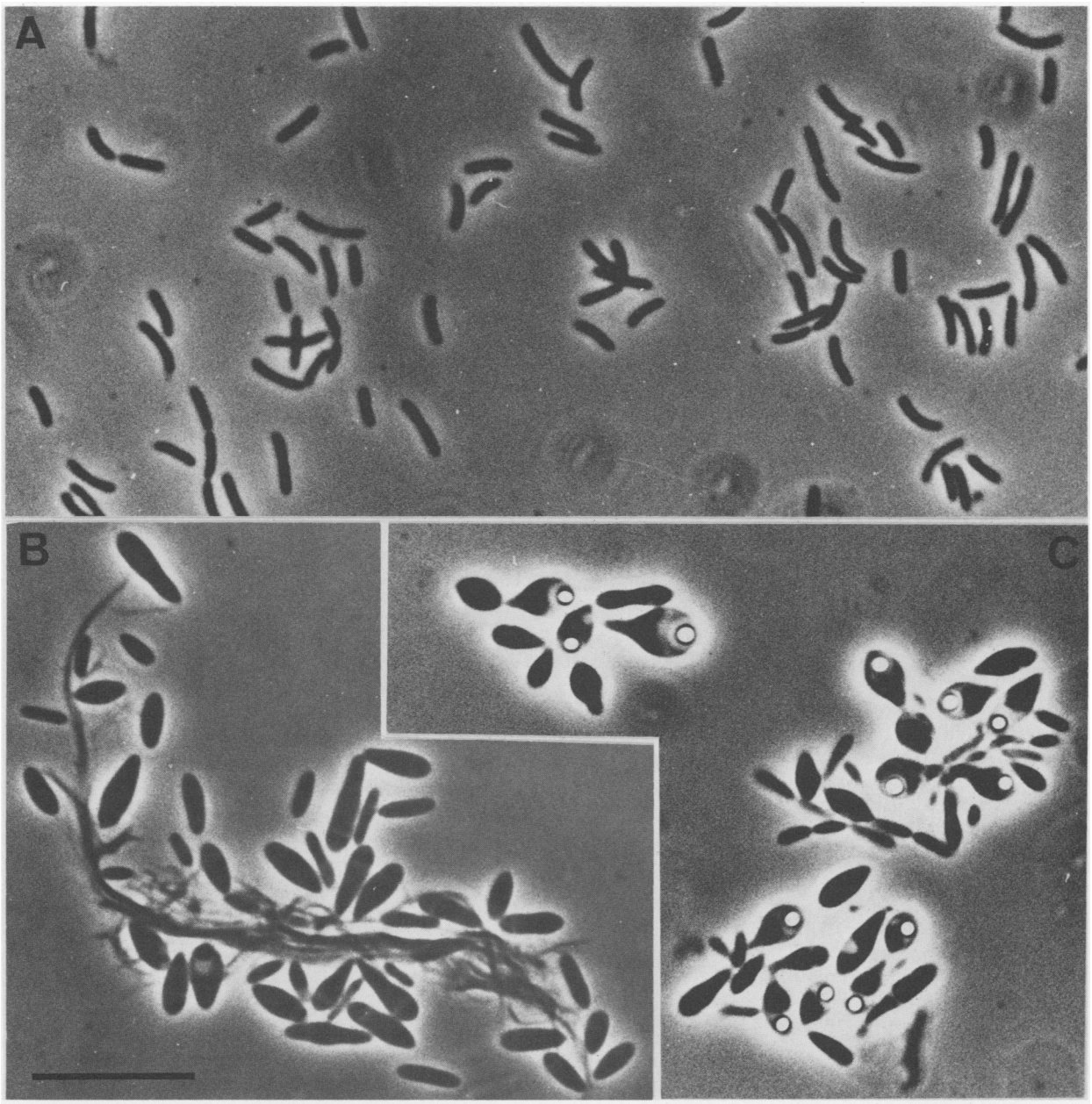

FIG. 2. Phase-contrast photomicrographs of cellulolytic isolates from wetwood (wet mount preparations). All strains were grown in RFT-CB broth for 24 h at 30°C. Panel A, Strain JW2; panel B, strain JW3. Cells are entangled in cellulose fibers that were introduced into the RFT-CB broth along with the inoculum. Panel C, Strain JW4. Note refractile spores within cells. All micrographs are at the same magnification. Bar = 10 μm.

4 μm. Cells were peritrichously flagellated as determined by electron microscopy of cells negatively stained with phosphotungstic acid. Spores were never observed in cultures of this strain. Attempts to induce sporulation (limiting the concentration of the growth substrate, heating cultures, or adding $CaCO_3$) were unsuccessful. Cells of strain JW3 (Fig. 2B) were motile and rod- to spindle-shaped, with an average length of 4.5 μm. Strain JW3 formed round terminal spores which swelled the sporangium. Cells of strain JW4 (Fig. 2C) were similar in morphology to those of strain JW3.

Cells of all three strains stained gram negative. Electron micrographs of thin sections of strains JW2 (Fig. 3A) and JW3 (Fig. 3B) showed that the cytoplasmic membrane was surrounded by a thin, densely stained inner layer and a diffuse, less densely stained outer layer. The ultrastructure of the cell wall resembled that of certain gram-negative, mesophilic, cellulolytic clostridia (12) and other gram-negative and gram-variable clostridia which also have a two-layered cell wall (25).

Growth and nutrition. All strains of cellulolytic bacteria isolated from wetwood were obligately anaerobic. Cellulose in medium RFT-C appeared to be completely utilized in 7 to 10 days, leaving a yellow-tinged cell pellet at the bottom of the tube.

Strain JW2 was selected for further characterization. This isolate fermented not only cellulose and cellobiose but also

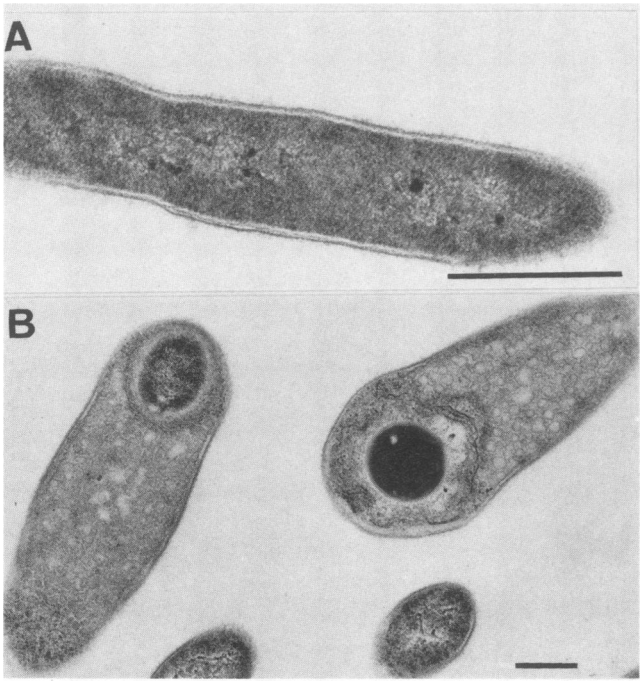

FIG. 3. Thin sections of strain JW2 cell (A) and of sporulating cells of strain JW3 (B) stained with uranyl acetate and Reynolds lead citrate solution. Both strains were cultured in RFT-CB broth for 24 h at 30°C. Bars = 0.5 μm.

xylan, D-glucose, L-arabinose, D-xylose, and glycerol. Other substances tested but not fermented include amino acids, carboxymethylcellulose, esculin, D-fructose, D-galactose, D-galacturonate, inulin, lactose, maltose, D-mannose, polygalacturonate, D-ribose, sodium pyruvate, L-sorbose, starch, sucrose, and D-trehalose. Strain JW2 fermented a wide range of cellulosic substrates, including ball-milled filter paper, tissue paper, alpha-cellulose, microcrystalline cellulose (Avicel), Solka-Floc, and cotton. In defined media (MJ-C and MJ-CB), strain JW2 required only *p*-aminobenzoic acid and biotin as growth factors for the fermentation of cellulose or cellobiose. Media supplemented with these growth factors sustained growth through at least five serial transfers.

For comparison, we determined the growth factor requirements of two other mesophilic cellulolytic bacteria, strain C7 (12) and *C. papyrosolvens* (13). Both strains also required *p*-aminobenzoic acid and biotin as growth factors for the fermentation of cellulose. *C. papyrosolvens* required only *p*-aminobenzoic acid to ferment cellobiose, whereas strain C7, like strain JW2, required both *p*-aminobenzoic acid and biotin to ferment this sugar.

Nongaseous fermentation products. Strain JW2 produced acetate, ethanol, and lactate from cellulose.

DNA base composition. The guanine-plus-cytosine content of the DNA of strain JW2 was 33.7 mol%.

DISCUSSION

With only two exceptions, cellulolytic activity was confined to the inner section of the wood core samples (Table 1). This observation indicates that cellulolytic bacteria were not introduced into enrichment cultures as contaminants but were present in the wetwood portion of the core. Cellulolytic bacteria in liquid from the core holes probably originated in the wetwood of the trees, inasmuch as the liquid is the result of the wetwood infection (5) and contains bacteria morphologically and physiologically similar to those found in wetwood (22, 28).

Strain JW2 cannot be placed in the genus *Clostridium* because apparently it does not form endospores. However, strain JW2 closely resembles strain C7, a freshwater cellulolytic clostridium (12), and *C. papyrosolvens*, a cellulolytic bacterium from brackish water (13). Gram reaction, cell envelope fine structure, and fermentation products are the same in all three strains. The guanine-plus-cytosine content of the DNA of strain JW2 (33.7 mol%) is similar to that of strain C7 (32.7 mol%) and *C. papyrosolvens* (30.0 mol%). Strain JW2 differs from cellulolytic members of the family *Bacteroidaceae* (9, 17, 19) primarily because it does not have a tripartite cell wall structure (Fig. 3A). Most likely, the other wetwood isolates, spore-forming strains JW3 and JW4, are species of *Clostridium*.

In a study which involved the enumeration of bacteria from wetwood, Schink et al. (22) found no cellulolytic bacteria among the 43 most frequently isolated anaerobic heterotrophs. Possible explanations for this observation are (i) a medium that selected for cellulolytic bacteria was not used, (ii) cellulolytic bacteria occur infrequently in large numbers in wetwood, or (iii) cellulolytic bacteria may associate closely with cellulose fibers in the wetwood samples (see Fig. 2B). Thus, the last positive dilution tube of a series enumerating heterotrophic bacteria would not be likely to contain cellulolytic bacteria.

No attempts were made in the present study to enumerate cellulolytic microorganisms in wetwood. The results of enumeration attempts would not have been meaningful because, as discussed above, it is likely that cellulolytic bacteria adhere to or are entangled in cellulose fibers in wetwood. Moreover, growth requirements and physiological characteristics of the diverse cellulolytic bacteria in wetwood are not known. Scanning electron micrographic observations of wetwood of poplar trees by Sachs et al. (20) have shown that vessel-ray pit membranes of plant cells near the sapwood-to-wetwood transition zone appear coated with bacteria and slightly eroded, and membranes closer to the pith are mostly destroyed. Since these structures contain cellulose (4), cellulolytic bacteria may play an important role in their destruction in wetwood. Also, wetwood has been shown to be significantly weaker than sapwood in strength properties (2, 3, 7), suggesting the breakdown of the structural cellulose of the cell walls. However, this has not been found in all cases (1, 5). Thus, it is not known whether cellulolytic bacteria play a major role in establishing wetwood. Possibly, cellulolytic bacteria attack cell wall cellulose and hemicelluloses after an initial infection by other microorganisms.

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